

STAPHYLOCOCCUS AUREUS COWAN I AND BRANHAMELLA CATARRHALIS AS  
B LYMPHOCYTE MITOGENS: CULTURE CONDITIONS FOR OPTIMAL DNA  
SYNTHESIS AND SELECTIVE STIMULATION OF HUMAN B LYMPHOCYTES

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## ABSTRACT

*Staphylococcus aureus* Cowan I and *Branhamella catarrhalis* that selectively stimulate human B lymphocytes to increased DNA synthesis with serum-free culture media were shown to do so also with culture media containing human serum or albumin when cultured for 3 days. After this time the transformed cells expressed very little or no surface bound immunoglobulin and did not form rosettes with sheep erythrocytes. When lymphocytes were cultured with the bacterial mitogens in the presence of serum or albumin for more than 3 days transformed T cells appeared. The bacteria induced antibody production, mainly of IgM class, in lymphocytes cultured in albumin supplemented medium.

## INTRODUCTION

Formalin-treated bacteria were earlier shown to induce increased DNA synthesis and polyclonal antibody production in human B lymphocytes (Banck and Forsgren, 1978). In that study serum-free culture medium was used. For optimal culture conditions serum or other additives are often necessary. Therefore it was investigated if *S. aureus* Cowan I and *B. catarrhalis* are selective B lymphocyte mitogens also in the presence of human serum or human albumin. Optimal condition were defined for B lymphocyte stimulation by bacteria in the presence of these additives.

## MATERIALS AND METHODS

### Lymphocytes and culture conditions

Blood was collected from healthy donors with heparin 10 IU/ml and was diluted with an equal amount of RPMI 1640 (Gibco Biocult Ltd., Scotland). Lymphocytes were prepared by density gradient centrifugation on Ficol-Isopaque (Lymphoprep, Nyeagaard AS, Norway). The cells were washed twice in RPMI 1640 and adjusted to a concentration of  $3 \times 10^6$  lymphocytes/ml in RPMI 1640 with gentamicin 24  $\mu\text{g}/\text{ml}$  and glutamine 4 mmol/ml. The culture medium was supplemented, as described in the result section, either with inactivated human

AB serum, human albumin (albumin 20 % for intravenous infusion, AB Kabi, Sweden) or transferrin (Sigma, Saint Louis, Miss.). For studies of DNA synthesis triplicate samples were cultured in microtitre plates with U-shaped wells (Linbro Scientific Inc., Hamden, Conn.). To each well was added 100  $\mu$ l of the lymphocyte suspension and 100  $\mu$ l of mitogen in RPMI 1640. The plates were incubated in 5 % CO<sub>2</sub> at 37°C. After 48 h was added 0.5  $\mu$ Ci of <sup>3</sup>H-thymidine (NET-027Z, specific activity 48 Ci/mmol, or NET-027 specific activity 6.7 Ci/mmol, New England Nuclear, Boston, Mass.) in 50  $\mu$ l of RPMI 1640. After pulsing for 22 - 24 h the cultures were harvested onto glass fibre filters with a Skatron harvesting machine (Skatron AS, Lierbyen, Norway). The filters were dried and transferred to scintillation vials containing 4 ml of Insta-Fluor solution (Packard) and the radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Results are presented as counts per minute (CPM). For studies of surface markers on transformed lymphocytes 5 x 10<sup>6</sup> lymphocytes in 5 ml culture medium were incubated in 50 ml plastic bottles (A/S Nunc., Denmark) in 5 % CO<sub>2</sub> at 37°C.

#### Preparation of T lymphocytes

Lymphocyte rosetting with sheep erythrocytes was performed according to Jondal et al. (1972). The lymphocytes were then gently layered on Lymphoprep and centrifugated. The rosetting cells were collected from the bottom of the tubes and the sheep erythrocytes were removed by osmotic shock and finally the lymphocytes were washed in RPMI 1640. In most experiments this procedure was repeated once.

#### B lymphocyte deficient patients

For some experiments were used lymphocytes from 2 boys with congenital X-linked hypogammaglobulinemia and a man, 50 years of age, with hypogammaglobulinemia of unknown etiology. All three had severe pan-hypogammaglobulinemia, no detectable bone-marrow plasma cells or blood lymphocytes with membrane immunoglobulin.



### Immunoglobulin determinations

For the determination of immunoglobulin production  $5 \times 10^6$  lymphocytes were cultured in plastic bottles (A/S Nunc., Denmark) in 5 ml of RPMI 1640 supplemented with 0.5 % human albumin, glutamin 2 mmol/ml and gentamicin 12  $\mu\text{g}/\text{ml}$  for 6 days. The IgG and IgM concentrations of the culture supernatants were determined by a double antibody inhibition radioimmunoassay according to Platts-Mills and Ishizaka (1975). In brief 50  $\mu\text{l}$  culture supernatant and 2-fold dilutions of control IgG and IgM (WHO standard serum) were separately added to 100  $\mu\text{l}$  rabbit-anti-human IgG and IgM. One hundred  $\mu\text{l}$  of  $^{125}\text{I}$ -labelled human IgG and IgM were added after 3 h and after a further 3 h 100  $\mu\text{l}$  of goat anti-rabbit IgG was added. After 15 h at  $4^\circ\text{C}$  the precipitate was washed and the radioactivity in the precipitate was counted. Values were obtained by the WHO standard sera.

### Mitogens

PHA (Wellcome Ltd., England) was used in final concentrations of 5, 2.5, and 1.25  $\mu\text{g}/\text{ml}$ . *S. aureus* Cowan I and *B. catarrhalis* were cultured and killed with formaldehyde as previously described (Banck and Forsgren, 1978). Final concentrations of 1/1000, 1/2000, 1/4000 were used. Triplicate samples were set up for each concentration and the median value for the dilution giving the highest thymidine incorporation was used.

### Detection of surface markers on transformed cells

For lymphocytes incubated with the bacterial mitogens the numbers of lymphoblasts with receptors for sheep erythrocytes was determined according to Jondal et al. (1972), and the number expressing surface immunoglobulin was determined by direct and indirect immunofluorescence. After incubation in serum-free RPMI 1640 at  $37^\circ\text{C}$  for 30 minutes and washing 3 times at  $37^\circ\text{C}$ , FITC-conjugated  $\text{F(ab')}_2$ -fragments of goat IgG against human light and heavy chains (Kallestad Inc., Minn.) was added. For indirect immunofluorescence was used in the second step FITC-conjugated  $\text{F(ab')}_2$  rabbit IgG against goat IgG (Cappel Laboratories, Cochranville, Pa.).

### Cryopreservation of lymphocytes

Lymphocytes adjusted to  $4 \times 10^6$  in RPMI 1640 with 20 % serum were placed on ice and dimethylsulfoxide was slowly added to a final concentration of 8 %. Freezing was performed with a Planar Mini-Freezer (Planar Products Ltd., England) at a rate of  $1^{\circ}\text{C}$  per minute. The cells that were stored in liquid nitrogen were thawed quickly and fresh medium was slowly added in great excess. The cells were washed and allowed to recover at  $37^{\circ}\text{C}$  for 1 h. Viability tested by trypan blue exclusion was above 85 %.

### RESULTS

#### The capacity of serum to support the growth of bacteria-stimulated lymphocytes

Higher thymidine incorporation in unstimulated lymphocytes was found with fetal calf serum (FCS) compared to human serum. The reason was that FCS induced lymphocyte transformation. Therefore FCS was not further tested.

Human serum did not induce lymphocyte transformation. The capacity to support the growth of mitogen-stimulated lymphocytes differed between sera from different donors. The sera therefore were compared to a reference serum before use. Either a pool of supporting sera or supporting serum from a single donor was used. Increasing the serum concentration above 5 % did not result in further increased thymidine incorporation when the lymphocytes were stimulated by PHA or the bacterial mitogens. In the case of lymphocytes from the same donor different batches of RPMI 1640 with and without 5 % human serum were compared and one representative experiment with *S. aureus* Cowan I is shown in Table 1. When some batches of RPMI 1640 (both from Flow and Gibco) were used without serum there was none or very little stimulation, for example batch Gibco U 69 1009 in Table 1. Without serum the results were highly variable for different batches of RPMI 1640. This difference was eliminated with 5 % human serum that also diminished the difference between triplicate samples that mostly were within  $\pm 15$  % of the mean. The optimal amount of mitogen was not changed by serum.



### The capacity of human albumin and transferrin to support the growth of bacteria-stimulated lymphocytes

Batches of RPMI 1640 that gave very low thymidine incorporation in lymphocytes stimulated by PHA and the bacterial mitogens were fully restored by the addition of 0.5 % albumin. When albumin 0.5 % was compared with 5 % pooled human serum the thymidine incorporation was somewhat higher in the presence of albumin compared to serum both in unstimulated lymphocytes and in lymphocytes stimulated by the bacterial mitogens. According to recent reports the supporting effect is due to transferrin that is carried by albumin (Dillner-Centerlind et al., 1979). When transferrin was compared to albumin it was found that the level of thymidine incorporation in mitogen-stimulated lymphocytes was somewhat lower with transferrin. However, as background levels were also lower the stimulation index was the same. Results were also compared with Iscoves modification of Dulbeccos medium (Gibco Europe, Glasgow, Scotland) which is a completely synthetic medium that can be used without serum. This medium was not superior to RPMI 1640 supplemented by serum, albumin or transferrin.

### Number of transformed lymphocytes

In 10 experiments were determined the number of transformed cells after 3 days of lymphocyte incubation with *S. aureus* Cowan I in the presence of serum. The mean percentage of cells with the morphology of a plasmablast in Giemsa-stained cytocentrifuge preparations was 7.4 % (range 4-12 %). With albumin instead of serum the results were identical. On prolonged incubation of lymphocytes with *S. aureus* Cowan I in the presence of serum there was a successive increase in the percentage of transformed cells from 7.4 % after 3 days to about 30 % after 6 to 7 days. Similar results were obtained when the culture medium was supplemented by albumin.

### Characterization of transformed lymphocytes

In Giemsa-stained cytocentrifuge preparations the transformed lymphocytes resembled plasmablasts. They were rich in strongly basophilic cytoplasm with many vacuoles. The nucleus was round or oval, it contained giant nucleoli

and was situated eccentrically. The size of the transformed cells was 3 to 4 times that of a small lymphocyte. With direct and indirect immunofluorescence no transformed cells with membrane immunoglobulin were detected.

The percentage of transformed cells that formed rosettes with sheep erythrocytes was determined in 6 experiments in which lymphocytes were incubated for 3 to 7 days with *S. aureus* Cowan I in the presence of serum. The mean percentage of transformed cells forming rosettes with sheep erythrocytes after 3 days was 4.7 % (range 0-7 %). With prolonged lymphocyte incubation the number of rosette-forming transformed cells successively increased to about 40 % of the transformed cells after 7 days. In these experiments the total number of lymphocytes was almost unchanged from day 3 to day 7.

Unfractionated lymphocytes were incubated with 50 % fresh medium and 50 % filtered supernatants from 7 days cultures of *S. aureus* Cowan I and *B. catarrhalis*. After 3 days thymidine incorporation increased to 10 to 20 times that of lymphocytes incubated with supernatants from unstimulated cells. Purified T lymphocytes and the lymphocytes from a boy with Bruton's disease were also stimulated by the lymphocyte supernatants. The supernatants of unstimulated lymphocytes or of bacteria incubated with culture medium only did not stimulate the lymphocytes. When lymphocytes from 3 patients with hypogammaglobulinemia lacking B lymphocytes were cultured together with *S. aureus* Cowan I and *B. catarrhalis* for 3 days in the presence of serum or albumin no significant response was detected. The response to PHA was the same as that of lymphocytes from healthy controls.

#### Reproducibility of lymphocyte stimulation with *S. aureus* Cowan I and *B. catarrhalis* and results with cryopreserved cells

The thymidine incorporation was compared for 5 different batches of *S. aureus* Cowan I. There was little difference in thymidine incorporation with different batches (Table 2). In Fig. 1 is shown the results of a representative experiment when the lymphocytes of the same donor were cultured on



6 different occasions with *S. aureus* Cowan I and PHA for 3 days.

Cryopreserved lymphocytes could be stimulated by the bacterial mitogens to increased thymidine incorporation. The degree of thymidine incorporation was almost as good as with fresh lymphocytes.

#### Immunoglobulin production

In Table 3 is shown the IgM and IgG concentrations of supernatants from lymphocytes of 3 healthy donors. There was a significant production of IgM in *B. catarrhalis* stimulated lymphocytes compared to non-stimulated lymphocytes. The IgG levels of supernatants from stimulated lymphocytes were slightly elevated compared to non-stimulated lymphocytes.

#### DISCUSSION

*S. aureus* Cowan I was tested as a B lymphocyte mitogen because of its protein A that was proposed to stimulate the B cells by cross-linking surface bound IgG (Forsgren et al., 1976). Serum-free medium was used in this initial study to avoid the interaction of serum IgG with the lymphocyte Fc-receptors and protein A. According to Sakane and Green (1978) soluble protein A in the presence of serum is a T cell mitogen and therefore there has been a suspicion that when *S. aureus* Cowan I is used in the presence of serum it may also stimulate T cells. All subsequent studies in which bacteria were used as B cell mitogens therefore have been performed with serum-free culture media. However, with the exception of the newly introduced Iscoves modification of Dulbeccos medium, lymphocyte culture media are recommended to be used with the addition of serum. When studying mitogen-induced antibody production immunoglobulin-free additives are needed. FCS, that is often used, was found unsuitable as it turned out to be mitogenic by itself. In fact, it has earlier been shown to contain B lymphocyte mitogenic factors (Melchers et al., 1975). In this study was shown that in the presence of serum or albumin *S. aureus* Cowan I and *B. catarrhalis* do not stimulate T lymphocytes provided that the lymphocytes are not cultured for

more than 3 days. During prolonged incubation also T lymphocytes are stimulated, probably due to the release of some mitogenic factor. This effect does not seem to be due to the release from the bacteria of protein A or other mitogenic substances as the supernatants of bacteria incubated in culture medium were not stimulatory.

The "null" cells, which have been proposed to be precursor cells of B cells (Haegert and Coombs, 1979) do not seem to be transformed by the bacterial mitogens as the lymphocytes of 2 patients with congenital X-linked hypogammaglobulinemia could not be stimulated to increased DNA synthesis by the bacterial mitogens and such patients have been shown to have "null" cells (Dhaliwal et al., 1980).

In albumin supplemented culture medium lymphocytes stimulated by *B. catarrhalis* were shown to produce IgM and possibly IgG antibodies. However, most IgG antibodies probably were shed from Fc-receptors of lymphocytes and monocytes as no warm incubations and washings of the cells were done before the cultures were set up.

*S. aureus* Cowan I and *B. catarrhalis* seem to be good mitogens for the in vitro study of B lymphocyte function by several reasons. They are cheap, easy to handle and produce. Results are reproducible and B lymphocytes can be selectively stimulated without the influence of monocytes (Banck and Forsgren, 1978) or helper T cells (Sirianni et al., 1979). Immunoglobulins of all classes are produced by lymphocytes stimulated by *S. aureus* Cowan I (Pryjma et al., 1980).

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TABLE 1.

Thymidine incorporation (CPM) in *S. aureus* Cowan I stimulated lymphocytes. Comparison between different batches of RPMI 1640 with and without 5 % pooled human serum

| Batch of RPMI 1640 | <i>S. aureus</i> Cowan I |           |
|--------------------|--------------------------|-----------|
|                    | No serum                 | 5 % serum |
| Gibco K 48 26 04   | 4 900                    | 13 300    |
| Gibco L 68 18 08   | 12 200                   | 14 500    |
| Gibco U 98 45 05   | 8 000                    | 15 900    |
| Gibco U 69 10 09   | 800                      | 14 300    |
| Flow 12 31 029     | 8 600                    | 13 100    |

TABLE 2.

Comparison of thymidine incorporation (CPM) in lymphocytes cultured for 3 days in the presence of 5 % human serum and *S. aureus* Cowan I from 5 different batches

| Batch | Lymphocyte donor I | Lymphocyte donor II |
|-------|--------------------|---------------------|
| 1     | 62 500             | 25 500              |
| 2     | 81 000             | 31 300              |
| 3     | 63 700             | 23 100              |
| 4     | 74 100             | 28 200              |
| 5     | 57 000             | 22 700              |

TABLE 3.

IgM and IgG levels ( $\mu\text{g/ml}$ ) in 6 days cultures with *B. catarrhalis*

| Lymphocyte<br>donor | IgM        |                       | IgG        |                       |
|---------------------|------------|-----------------------|------------|-----------------------|
|                     | No mitogen | <i>B. catarrhalis</i> | No mitogen | <i>B. catarrhalis</i> |
| I                   | < 30       | 440                   | 340        | 490                   |
| II                  | < 30       | 1 750                 | 890        | 900                   |
| III                 | 70         | 460                   | 460        | 850                   |



Fig. 1. DNA-SYNTHESIS AFTER 3 DAYS OF LYMPHOCYTE INCUBATION WITH PHA AND *S. AUREUS* COWAN I WHEN LYMPHOCYTES FROM THE SAME DONOR WERE TESTED REPEATEDLY.



